sAPP and sAPP increase structural complexity and E/I input ratio in primary hippocampal neurons and alter Cahomeostasis and CREB1-signaling

sAPPβ and sAPPα increase structural complexity and E/I input ratio in primary hippocampal neurons and alter Ca^{2+} homeostasis and CREB1-signaling

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Abstract

One major pathophysiological hallmark of Alzheimer's disease (AD) is senile plaques composed of amyloid β (Aβ). In the amyloidogenic pathway, cleavage of the amyloid precursor protein (APP) is shifted towards Aβ production and soluble APPβ (sAPPβ) levels. Aβ is known to impair synaptic function; however, much less is known about the physiological functions of sAPPβ. The neurotrophic properties of sAPPα, derived from the non-amyloidogenic pathway of APP cleavage, are well-established, whereas only a few, conflicting studies on sAPPβ exist. The intracellular pathways of sAPPβ are largely unknown. Since sAPPβ is generated alongside Aβ by β-secretase (BACE1) cleavage, we tested the hypothesis that sAPPβ effects differ from sAPPα effects as a neurotrophic factor. We therefore performed a head-to-head comparison of both mammalian recombinant peptides in developing primary hippocampal neurons (PHN). We found that sAPPα significantly increases axon length ($p = 0.0002$) and that both sAPPα and sAPPβ increase neurite number ($p < 0.0001$) of PHN at 7 days in culture (DIV7) but not at DIV4. Moreover, both sAPPα- and sAPPβ-treated neurons showed a higher neuritic complexity in Sholl analysis. The number of glutamatergic synapses ($p < 0.0001$), as well as layer thickness of postsynaptic densities (PSDs), were significantly increased, and GABAergic synapses decreased upon sAPP overexpression in PHN. Furthermore, we showed that sAPPα enhances ERK and CREB1 phosphorylation upon glutamate stimulation at DIV7, but not DIV4 or DIV14. These neurotrophic effects are further associated with increased glutamate sensitivity and CREB1-signaling. Finally, we found that sAPPα levels are significantly reduced in brain homogenates of AD patients compared to control subjects. Taken together, our data indicate critical stage-dependent roles of sAPPs in the developing glutamatergic system *in vitro*, which might help to understand deleterious consequences of altered APP shedding in AD patients, beyond Aβ pathophysiology.

Keywords: sAPP, AD, neuronal plasticity, neurodevelopment, CREB-1 signaling

Research highlights:

- sAPPα and sAPPβ have a critical stage-dependent role in the developing glutamatergic system *in vitro*
- sAPPβ impacts in a different manner on the developing glutamatergic system compared to sAPPα
- Effects of sAPPα are associated with CREB-1 signaling

List of abbreviations:

Aβ, amyloid β; AD, Alzheimer's disease; ADAM, A Disintegrin And Metalloproteinase (α-secretase); APP, amyloid precursor protein; AMPA-R, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BACE, β-site of APP cleavage enzyme (β-secretase); BDNF, brain-derived neurotrophic factor; CNS, central nervous system; CREB, cAMP-response element binding protein; CSF, cerebrospinal fluid; DIV, days in vitro; EGFP, enhanced green fluorescent protein; GABA, γ-Aminobutyric acid; HRP, horse-radish peroxidase; LOAD, late-onset Alzheimer's disease; LSM, laser-scanning-microscope; LTP, long-term potentiation; NMDA-R, *N*-methyl-D-aspartate receptor; PCR, polymerase chain reaction; PHN, primary hippocampal neuron; PSD, postsynaptic density; sAPP, soluble amyloid precursor protein; TEM, transmission electron microscopy

Introduction

Besides being a central molecule in Alzheimer's disease (AD) pathophysiology, amyloid precursor protein (APP) and its cleavage products are known to be major players in establishing and maintaining neuronal architecture (Nicolas and Hassan, 2014). Depending on whether α- or β-secretase (BACE1) cleaves first, subsequent APP cleavage by γ-secretase fosters or precludes the generation of the neurotoxic amyloid β (Aβ) fragment (Prox et al., 2012). Alongside failure of Aβ clearance mechanisms and missense mutations in presenilin 1 or 2 genes, a shift in proteolytic APP processing towards BACE1 cleavage in AD patients that leads to gradually rising Aβ42 levels in the brain and therefore causing neuronal death is a well described hypothesis (Selkoe and Hardy, 2016). Besides the increased Aβ42 and soluble APPβ (sAPPβ) production, less sAPPα is also generated.

In contrast to the deleterious effects of Aβ on neurons, the soluble APPα cleavage product (sAPPα) is known to have neurotrophic or synaptic plasticity enhancing characteristics, as shown by increase of long-term potentiation (LTP) (Taylor et al., 2008). sAPPα, but also sAPPβ levels seem to be decreased in cerebrospinal fluid (CSF) of AD patients (Colciaghi et al., 2004; Hock et al., 1998; Lannfelt et al., 1995). BACE1 inhibition, which is a potential disease-modifying therapy for AD, leads to decreased sAPPβ levels in the CSF of treated subjects (Kennedy et al., 2016). Interestingly, a missense mutation within the gene encoding for the major α-secretase, ADAM10, resulting in attenuated activity has been recently associated with late-onset AD (LOAD) (Suh et al., 2013). Both findings can therefore lead to an imbalance of soluble APP cleavage products in the central nervous system (CNS). Until now, it has not been known how APP influences neural development and adult brain function or whether loss of these functions can account for AD pathophysiology. Hence, deciphering the physiological functions of both sAPP forms is of crucial importance.

In neuronal cell culture, it has been shown that sAPPα interacts with the p75 neurotrophin receptor to stimulate neurite outgrowth (Hasebe et al., 2013). Furthermore, sAPPα and sAPPβ decrease cell adhesion and thereby decrease dendrite outgrowth and increase axon outgrowth (Chasseigneaux et al., 2011). Neuroprotective effects are also described for sAPPα as it could be shown that it protects hippocampal neurons against Aβ induced oxidative injury (Goodman and Mattson, 1994) and proteasomal stress (Copanaki et al., 2010). Moreover, sAPPα has been shown to extenuate established synaptic and cognitive deficits in the APP/PS1ΔE9 AD mouse model (Fol et al., 2015). Another study generated a sAPPα-knock-in mouse to show that sAPPα is sufficient to rescue the abnormalities of APP-deficient mice, including reductions in brain weight and the impairment in spatial learning and LTP (Ring et al., 2007). Conversely, there is

one study published demonstrating that cleavage of sAPPβ under trophic-factor deprivation generates a cleavage product that is able to induce death receptor 6 signaling and thereby lead to cellular self-destruction (Nikolaev et al., 2009).

Generally, effects of sAPPα on neurons are well established, whereas much less is known about sAPPβ function (Chasseigneaux and Allinquant, 2012). In addition, the intracellular pathways mediating the sAPP functions are also largely unknown. Since sAPPβ derives from amyloidogenic APP cleavage, we hypothesize that sAPPβ opposes neurotrophic sAPPα effects. Therefore, to further evaluate the roles of both sAPPs in neuronal development, we performed in this study a head-to-head comparison in developing primary hippocampal neurons at different time stages.

Materials and Methods

Synthesis of recombinant sAPP peptides

sAPPα and sAPPβ gene fragments were amplified by PCR with human APP695 cDNA as template. sAPPs were then N-terminally tagged with the APP secretory sequence, 8x histidine tag, and EGFP. Constructs were subcloned in pcDNA ™5/FRT (Invitrogen, Carlsbad, CA, USA) vector by NheI and ApaI restriction sites. The same construct (empty EGFP vector), without sAPP sequence (APP secretory sequence, 8x histidine tag, and EGFP), was used as a control peptide. For purification of sAPP and control proteins, Flp-In™HEK293 cells were stably transfected (Flp-In™ system, Invitrogen, Carlsbad, CA, USA) with sAPPα, sAPPβ and the EGFP construct. Ni-NTA charged agarose beads (Qiagen Hilden, Germany, #30210) were added to the supernatant of the cultivated cells containing the his-tagged sAPP/EGFP fusion proteins. After overnight binding, beads were washed three times with low imidazol buffer (40 mM imidazol) and sAPP/EGFP fusion proteins were subsequently eluted in high imidazol buffer (150 mM imidazol). For buffer exchange and protein concentration eluates were pooled and concentrated via 100 kDa cut-off columns (Millipore Darmstadt, Germany, #UFC810024). Proteins were recovered in PBS and analyzed qualitatively and quantitatively via SDS-PAGE, Western blot, BCA assay, and SimplyBlue™ SafeStain (Invitrogen, Carlsbad, CA, USA) (Fig. S1).

Lentiviral plasmid design and vector production

To generate infectious lentiviral particles, sequences of sAPPα and sAPPβ were cloned into pUltra Hot vector. sAPPα and sAPPβ gene fragments were amplified by PCR with the above-mentioned sAPPα/β pcDNA ™5/FRT as template and inserted into pUltra Hot vector by AgeI and NheI restriction sites. The expressed proteins were N-terminal fusion proteins with mCherry. pUltra Hot is a lentiviral vector backbone for bi-cistronic expression of the gene of interest and the fluorescent reporter mCherry under the control of a human ubiquitin promoter. pUltra Hot was a gift from Malcolm Moore (Addgene plasmid # 24130) and served as a control plasmid to induce viral stress on neurons. psPAX2 is a packaging plasmid encoding HIV-1 gag/pol sequences under the control of a SV40 promoter. psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260). pMD2.G is an envelope-expressing plasmid encoding for VSV-G glycoprotein under the control of a CMV promoter. pMD2.G was a gift from Didier Trono (Addgene plasmid # 12259). For the virus production, LentiX 293T cells (Takara Clontech, Mountain View, CA, USA) were co-transfected (calcium phosphate transfection method) with pUltra Hot, psPAX2 and pMD2.G. Six hours after transfection, the medium was changed to remove transfection reagent in the conditioned medium to which the virus is secreted. 48h after

transfection conditioned medium was collected and filtered using a 0.2 µm sterile filter (Sarstedt, Nuembrecht, Germany). Conditioned medium was transferred to 38.5 ml Beckman Ultra-Clear™ tubes containing 3 ml 20% sucrose and spinned for 2.5 h at 4°C and 24,000 rpm in a Beckman SW32Ti swinging bucket rotor. Supernatant was discarded and virus was resuspended in DPBS, aliquoted and stored at -20°C until use.

Preparation of primary hippocampal neurons

Primary hippocampal neurons were prepared from brains of C57BL/6 embryonic mice (E18), as described previously (Kaech and Banker, 2006). Briefly, hippocampi were dissected from embryonic brains, the meninges were removed and the cells were dissociated by trypsinization (0.25%) for 20 min at 37 °C. The dissociated cells were resuspended in serum-free neurobasal medium (Gibco®, Invitrogen, Germany) supplemented with 10% B27 (Gibco®, Invitrogen, Germany) and 0.5 mM l-glutamine and seeded into poly-l-lysine-coated culture dishes at a density of $2.2x10^4$ cells per cm^2 for immunocytochemistry (grown on coverslips) and $6.6x10^4$ cells per cm^2 for cell lysis. After 45 min, medium was replaced completely by the same medium, to reduce astroglial growth. Cells were maintained at 37°C in the presence of 5% CO_2 and 10% O_2 in a humidified incubator.

Morphometric analysis of PHN

For morphometric analyzes of PHN, neurons were treated on DIV2 with 100 nM His-EGFP-sAPPα/β or the His-EGFP control peptide, fixed on DIV7 and stained with anti-MAP2 and anti-tau (For antibody specification see list below). To make sure that a substantial amount of sAPPs remains in the culture medium, we used 100nM His-EGFP-sAPP peptide to study long-term effects on neuronal culture. This concentration was previously tested by other groups in similar studies (Chasseigneaux et al., 2011). PHNs were fixed for 20 min in a 4% PFA solution in DPBS. Cells were then washed three times with PBS and subsequently permeabilized and blocked in Roti®-ImmunoBlock (Carl Roth, Karlsruhe, Germany) and 0.3% TritonX-100 for 1 h at RT. Incubation with primary antibodies was performed overnight at 4°C with gentle shaking. The next day, cells were washed three times for 10 min with PBS. Incubation with secondary antibodies was performed for 1 h at RT with gentle shaking. Then cells were washed three times for 10 min with DPBS and embedded in Mowiol® 4-88 plus DABCO. To determine primary axon length, entire neurite length and neurite number, TIFF images of stained neurons were analyzed by ImageJ pluginsoftware NeuronJ. The longest tau positive neurite was considered as the primary axon in DIV7 neurons and the longest beta-III-tubulin neurite was

considered as the primary axon in DIV4 neurons respectively. Sholl analysis (SHOLL, 1953) was performed semi-automatically using ImageJ pluginsoftware Concentric Circles. Briefly, a series of concentric circles was drawn around the soma with 20 µm between each circle. The number of intersections were counted manually and plotted against the distance from the soma. 180 neurons from three independent cultures were sampled for DIV4 and DIV7 respectively.

Determination of inhibitory and excitatory synapse number

To determine synapse number, PHNs were fixed and stained at DIV14 using the same procedure as described in Morphometric analysis of PHN in the Materials & Methods section. Earlier time points were not suitable for synapse number determination due to immaturity of neurons. Cells were labelled with the primary antibodies anti-Homer1 as postsynaptic marker (SynapticSystems, Göttingen, Germany, #160003), anti-Bassoon as presynaptic marker (Enzo Lifesciences, Lörrach. Germany, #VAM-PS003) and anti-MAP2 as dendritic marker (EnCor Biotechnology, Gainesville, USA, #CPCA-MAP2) upon lentiviral mCherry-sAPP overexpression (DIV2 transduction). The transduction efficiency was about 95% (data not shown). As sAPP-peptides were secreted into the supernatant upon lentiviral overexpression, untransduced neurons were also used for quantification. Colocalization of both synaptic markers along the neurite indicate a mature glutamatergic synapse. All of these are well established synapse and neurite markers used for similar assays (Goetz et al., 2006; Grabrucker et al., 2011; Wang et al., 2016). Confocal images of immunostained neuronal cultures were acquired with a magnification of 63x (water immersion objective, pinhole aperture 1 µm) by using a Zeiss LSM 710 Meta laser scanning microscope (Carl Zeiss, Jena, Germany), as described previously (Beyer et al., 2012). Quantification was performed semi-automatically by using ZEN blue edition (Zeiss, Oberkochen, Germany). The criteria for determing colocalization was an overlap of intensity peaks of homer1 and bassoon immunofluorescence staining. The counts of glutamatergic inputs were normalized to 10µm dendrite length (MAP2 staining) and are indicated as numbers in the results section.

Western blot analysis

Before lysis, neurons pre-incubated with His-EGFP-sAPP were briefly stimulated with 10 µM glutamate in wash buffer (for buffer composition see FLIPR-analysis part in Material & Methods section) for 30 s. After an additional 3 min in wash buffer, cells were lysed in brain-extraction buffer (BEX: 25 mM Tris pH 8.0, 20 mM NaCl, 0.6% desoxycholate, 0.6 % Igepal CA-630). Cell lysates (10-20 µg total protein, determined by BCA assay) were separated by

lithium dodecyl sulphate (LDS)-polyacrylamide gel electrophoresis (NuPage Novex Bis-Tris 4-12% gradient gels, Invitrogen) prior to electrophoretic transfer onto 0.2 µm pore size nitrocellulose membrane (GE Healthcare, Boston, USA, #10600001). The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature. Overnight incubation at 4°C was performed for primary antibody binding (for used antibodies see below). The next day, blots were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies prior to exposure to ECL Luminata Forte (Millipore, Darmstadt, Germany, #WBLUF0100). For detection of the corresponding non-phosphorylated proteins, blots were stripped with 0.1 M glycine, 1% SDS, 1% Tween-20 (pH 3.5) for 15 min at 37°C, followed by blocking in 5% non-fat dry milk and incubated with the antibodies corresponding to the total proteins as described above. Digitized images of the immunoblots were used for densitometric measurements with ImageJ. Relative levels of phosphorylated proteins were determined by normalization of the density of images from phosphorylated proteins with that of the total proteins on the same blot.

Antibodies

The following antibodies were purchased from commercial suppliers: Western blot: anti-phospho-CREB Ser^{133} (Cell Signaling Technology, clone 8763, #9198) (1:1,000), anti-phospho-p44/p42 MAPK (ERK1/2) Thr^{202}/Tyr^{204} (Cell Signaling Technology, clone E10, #9106) (1:1,000), anti-CREB (Cell Signaling Technology, clone 86B10, #9104) (1:1,000), anti-p44/p42 (ERK1/2) (Cell Signaling Technology, clone 86B10, #9104) (1:1,000), anti-beta III Tubulin (Abcam, #ab18207) (1:1,000). Immuncytochemistry (ICC): anti-VGlut1 (SynapticSystems, #135304) (1:400), anti-VGAT (SynapticSystems, #131011) (1:400), anti-Gephrin (SynapticSystems, #147011) (1:400), anti-Homer1 (SynapticSystems, #160003) (1:500), anti-Bassoon (Enzo Lifesciences, #VAM-PS003) (1:500), anti-MAP2 (EnCor Biotechnology, #CPCA-MAP2) (1:500), anti-beta III Tubulin (Neuromics, #MO15013) (1:500), anti-GAPDH (abcam, #ab9485). Western blot human samples: anti-human sAPPα (IBL International, clone 2B3, #11088), anti-human sAPPβ (IBL International, #18957). Secondary antibodies used for Western blot were HRP goat anti-mouse IgG (H+L) and HRP goat anti-rabbit IgG (H+L) (Invitrogen, 1:10,000). Secondary antibodies used for ICC were Alexa Fluor® 488/546/647 Goat anti-Rabbit/Mouse/Chicken IgG (H+L) (Invitrogen, 1:750).

Electron-microscopical investigations

Neurons were plated with a density of $6.6x10^4$ cells per cm^2. After 14 days in culture, neurons were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer with 1% sucrose (pH 7.3) and postfixed with 1% osmium tetroxide for 4 h. Cultures were then dehydrated through an increasing propanol series, stained with 2% uranyl acetate and embedded in epoxy resin (Epon 812, Fluka, Germany). Ultrathin sections (70–80 nm) were cut with a diamond knife on a Reichert ultramicrotome and collected on 300- mesh grids. The sections were stained with lead citrate and examined at a voltage of 100 kV by using the transmission electron microscope JEM-1400 (Jeol, Akishima, Japan). Images were recorded with a resolution of 2,048 × 2,048 pixels and a magnification of 50,000x using a Veleta digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany) and the iTEM software (Olympus Soft Imaging Solutions GmbH, Münster, Germany). At least 20 images of PSDs from asymmetric synapses in different cells were acquired per condition and independent experiment. Single images of the synapse were taken to measure PSD thickness. The thickness was determined manually by using ImageJ to measure the thickness from electron dense areas at three different locations in each PSD (central, and left/right from the centre, orthogonal to the PSD). The three measured values were averaged and defined as PSD thickness for the measured synapse.

FLIPR-analysis

Intracellular Ca^{2+} changes were monitored using the FLIPRTetra fluorometric imaging plate reader (Molecular Devices, CA, USA). Before dyeloading, Neurobasal culture medium was removed. The neurons were washed three times with imaging buffer (NaCl 145 mM, KCl 5 mM, $CaCl_2$ 2 mM, Glucose 25 mM, HEPES 12 mM, pH was adjusted to 7.4). After the last wash step, neurons were loaded with 2 µM Fluo-4 diluted in imaging buffer. Neurons were incubated for 45 min at 37°C. Then, neurons were washed three times with 180 µl imaging buffer left after the last wash step. Neurons were incubated for 15 min with 1nM His-EGFP-sAPPα, His-EGFP-sAPPβ or the control peptide His-EGFP. We decided to use 1 nM of both sAPP forms and control peptide in the FLIPR experiments, since this concentration (concentration response curve with 0.01, 0.1, 1 and 10 nM) was previously tested by other groups in similar studies on Ca^{2+} mobilization (Furukawa et al., 1996a; Furukawa and Mattson, 1998). Regarding long-term studies, to make sure that a substantial amount of sAPPs remains in the culture medium over the time course of the experiment, we used the supra-effective concentration of 100nM sAPP peptide to the study long-term effects of sAPPs on neuronal culture. This experiment was not aimed at defining exactly the minimal effective concentration, but rather to investigate mechanistically the long-term effects of sAPPs. Further, this

concentration was previously tested successfully by other groups in similar experiments (Chasseigneaux et al., 2011). After recording the baseline for 10 s, 20 µl imaging buffer containing 100 µM glutamate (final concentration in wells was 10 µM) was added to the wells and the kinetic of the Ca^{2+} responses was measured on the FLIPR device for evaluation of intrinsic compound activity.

Synaptoneurosome preparation

Synaptoneurosomes and homogenates were prepared as described in Tai et al. 2012 (Tai et al., 2012) using human end stage AD and control brain tissue provided by the Edinburgh Brain Bank. In brief, approximately 300 mg of Brodmann area 41/42 was homogenized in 1 ml of ice cold Buffer A (25 mM HEPES pH 7.5, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$), supplemented with 2 mM dithiothreitol (DTT), protease inhibitors (roche complete mini) and phosphatase inhibitors (Millipore, 524629). The homogenate was filtered through 2 layers of 80 µm nylon filter (NY8002500, Millipore), and a 200 µl aliquot of this was saved as the crude homogenate. The saved aliquot was mixed with 140 µl water, 60 µl of 10% SDS and boiled for 5 minutes. To prepare the synaptoneurosomes the remainder of the homogenate was filtered through a 5 µm filter (SLSV025NB, Millipore) and centrifuged at 1,000 g for 5 minutes. The supernatant was removed and the pellet was suspended in Buffer A and centrifuged again (1,000 g for 5 min). The supernatant was again removed and the resulting synaptoneurosome pellet was suspended in 400 µl of Buffer B (50 mM Tris [pH 7.5], 1.5% SDS, and 2 mM DTT) and boiled for 5 min.

Protein concentration was determined using a BSA assay (ThermoFisher, 23225) and 5 mg of protein from either synaptoneurosome or crude homogenate was loaded onto NuPAGE 4-12% Bis-Tris precast polyacrylamide 15 well gels (Invitrogen, Paisley, UK) along with molecular weight marker (Li-Cor, Cambridge, UK). Proteins were electro-transferred to nitrocellulose membrane (Bio-Rad, Hemel Hempstead, UK). Membranes were blocked using Odyssey Blocking Buffer (927-40000, LI-COR) diluted 1:1 in PBS. Primary antibodies were incubated overnight in block with gentle shaking. Proteins were visualized on an Odyssey infrared system using the appropriate 680 and 800 IR dye secondary antibodies (1:50,000, LI-COR Biosciences) and were analyzed using Odyssey software (LI-COR Biosciences). Patient demographic data are depicted in Table S1.

Data analysis

All statistical analyzes were carried out by GraphPad Prism 6.05. Data was tested for Gaussian distribution by the D'Agostino-Pearson omnibus normality test. For comparison of two groups, a Students *t*-test was used. When data was not normally distributed, the ranked values of two groups were tested for significant differences using a Mann-Whitney *U*-test. A Kruskal-Wallis test and Dunn's post hoc test for multiple comparisons were used when more than two groups were tested for significant differences. Statistically significant differences in FLIPR analysis among different groups were determined using Two-Way ANOVAs with a Dunnett's post-hoc test for multiple comparisons. The results are expressed as median (25^{th}–75^{th} percentile).

As an integrative analysis over different distances the results of the Sholl analysis were analyzed in comparative regression experiments (Dünkler et al., 2015). A global regression model fitted to the samples of both groups (treated/untreated) was tested against a pair of group-wise models fitted individually to the curves treated or the untreated samples. The significance of a difference between the treated group and the untreated group was determined by a comparison of the least square errors of the global model and the pair of individual models (F-Test) (Lomax et al., 2012; Motulsky and Ransnas, 1987). The model types were selected via the Akaike information criterion (Burnham et al., 2002). They were chosen from a set of 10 predefined model types. The corresponding software (CuCompare) is available at http://sysbio.uni-ulm.de/soft/CuCompare.

Results

sAPP treatment of PHN affects neuronal morphology and complexity in a stage-dependent manner

To determine the differential effects of sAPPs on neuronal morphology and branching we produced recombinant proteins of human sAPPα695 and sAPPβ695 in HEK293 cells. These bear all post-translational modifications observed in eukaryotic cells. PHNs were treated at DIV2 with 100 nM His-EGFP-sAPPα or His-EGFP-sAPPβ and fixed and immunostained with MAP-2 and Tau at DIV7 or β-III-tubulin at DIV4 respectively. His-EGFP-sAPP administration (100 nM) to PHN at DIV2 with fixation at DIV 4 did not lead to altered primary axon length (Fig. 1A), entire neurite length (Fig. 1B), and neurite number (Fig. 1C). The median lengths, 25th and 75th percentiles are depicted in table 1.

When PHNs were treated with the same concentration of His-EGFP-sAPP at DIV2 and fixed at DIV7, the median primary axon length was increased significantly by 21.5% upon His-EGFP-sAPPα treatment (p = 0.0002) but not upon His-EGFP-sAPPβ treatment (Fig. 1 D). Morphometric analysis also revealed a significant increase in median neurite length per neuron ($p < 0.0001$) (Fig. 1E) and also in median neurite number by 50% upon His-EGFP-sAPPα and 33.3% upon His-EGFP-sAPPβ treatment ($p < 0.0001$) (Fig. 1F). The median lengths, 25th and 75th percentiles are depicted in table 1. The significant increase in neurite number upon His-EGFP-sAPPα and His-EGFP-sAPPβ treatment raised the question if there is also alteration in neuritic ramification. To determine neuritic complexity at DIV7, Sholl analysis was performed. We plotted the number of intersections of neurites with each circle against the distance from the cell body (see scheme in Fig. 1G). This extensive analysis revealed a significant increase in neuritic arborization upon His-EGFP-sAPPα and His-EGFP-sAPPβ treatment (Fig. 1H) significant for distances from soma from 20 µm to 200 µm, $p < 0.05$). Further analysis revealed a distinct pattern of arborization for His-EGFP-sAPPα and His-EGFP-sAPPβ, with increased branching close to the soma for His-EGFP-sAPPα. Exemplary images of analysed neurons are shown in Fig. S2.

Table 1. Summarized morphological analysis of primary hippocampal neurons

	4 DIV ctrl	**4 DIV sAPPα**	**4 DIV sAPPβ**
Axon length [nm]	113.1 (86.14 – 146.5)	109.3 (77.41 – 139.2)	113.5 (35.19 – 153.8)
Neurite length [nm]	226.2 (182.8 – 268.4)	220.0 (174.3 – 277.6)	242.6 (191.8 – 295.6)

Neurite number	4 (3 – 5)	4 (3 – 5)	4 (3 – 5)

	7 DIV control	**7 DIV sAPPα**	**7 DIV sAPPβ**
Axon length [nm]	324.4 (255.2 – 438.5)	394.1 (322.6 – 470.7)*	352.2 (283.1 – 445.3)
Neurite length [nm]	1234.0 (868.8 – 1680.0)	1696.0 (1430.0 – 2174.0)*	1622.0 (1303.0 – 2095.0)*
Neurite number	12 (9 – 16)	18 (14 – 23)*	16 (12.25 – 22)*

Axon lengths, neurite lengths and neurite numbers are indicated as median (25^{th}-75^{th} percentile). Statistical significance is marked by an asterisk. *$p < 0.0001$, One-Way ANOVA, Kruskal-Wallis test, Dunn's post-hoc test.

Advanced glutamatergic synaptic architecture upon sAPP overexpression

To test the hypothesis that the increased neuritic arborization of PHN upon His-EGFP-sAPP treatment at DIV7 (first mature synapses observed) is associated with an increased function of glutamatergic synapses, we determined the synapse number upon lentiviral sAPP overexpression and compared them with control neurons at DIV14, where synaptic maturation is well established. Use of the lentiviral expression system ensured a stable and brief secretion of sAPP to supernatant of neuronal cell culture from DIV2 to DIV14. Secretion of sAPP peptides to neuronal cell culture supernatant was verified by Western blot (Fig. S1). Using double staining for Homer1 (postsynaptic marker) and Bassoon (presynaptic marker) at DIV 14, we were able to discriminate immature from mature glutamatergic synapses (Grabrucker et al., 2009). Colocalization of Homer1 and Bassoon indicated a fully developed excitatory synapse (Fig. 2A). We observed that 75% more mature glutamatergic synapses were developed until DIV14, when mCherry-sAPPα was overexpressed ($p < 0.0001$) and 25% more mature synapses when mCherry-sAPPβ ($p < 0.0001$) was stably secreted for 12 DIV in primary hippocampal neurons. The median number of mature glutamatergic synapses at DIV14 was 1.6 (1.2–2.0) in the control condition, 2.8 (2.1–3.5) upon mCherry-sAPPα overexpression and 2.0 (1.6–2.3) upon mCherry-sAPPβ overexpression (Fig. 2B). mCherry-sAPPα as well as mCherry-sAPPβ overexpression resulted in a marked rightward shift in the frequency distribution of synapse numbers, indicating an increased number of colocalizations (Fig. 2C).

To test the hypothesis that increased sAPP levels, leading to an increased number of glutamatergic synapses, also lead to alterations in the subsynaptic architecture, we determined PSD layer thickness from asymmetric synapses in mCherry-sAPP-overexpressing neurons by

electron microscopy. Asymmetric synapses were distinguished from symmetric synapses. The former are excitatory in function and are formed by axon terminals that contain spherical synaptic vesicles and an electron-dense PSD (Fig. 2D, asterisks mark PSDs). We observed that PSDs of neurons exposed to higher extracellular sAPPα levels showed a 27.4% increase in median layer thickness compared to control neurons. PSDs of mCherry-sAPPβ-overexpressed neurons were not significantly changed. The median PSD strength of neurons was 14.6 nm (10.5–17.6 nm) in control, 18.6 nm (14.1–23.0 nm) after treatment with mCherry-sAPPα and 14.0 nm (10.9–17.1 nm) after treatment with mCherry-sAPPβ (Fig. 2E). Increased exposure to mCherry-sAPPα led to a homogenous rightward shift in frequency distribution of PSD layer thickness (Fig. 2F).

Increased neuritic arborization is associated with higher levels of activated markers of neuronal activity

Since it has been previously described that sAPP levels are altered during aging and disease, we hypothesized that sAPPs can influence neuronal activity of maturated PHNs. To test, whether mature hippocampal neurons are responsive to sAPP effects mediated by the glutamatergic system in principal, neurons were loaded with a Ca^{2+}-sensitive dye (Fluo4) and incubated for 15 min with 1 nM of His-EGFP control peptide, His-EGFP-sAPPα or His-EGFP-sAPPβ at DIV14. Subsequent Ca^{2+}-imaging by a FLIPR system revealed that both His-EGFP-sAPPα and His-EGFP-sAPPβ administration increased glutamate (stimulation with 10 µM glutamate) induced cytoplasmatic $Ca2^{+}$ levels in mature hippocampal neurons (Fig. 3A).

Next we tested whether downstream Ca^{2+} signaling is also affected by long-term application of His-EGFP-sAPP peptides. We administered 100 nM His-EGFP control peptide, His-EGFP-sAPPα or His-EGFP-sAPPβ to primary hippocampal neurons at DIV2 (lentiviral mCherry-sAPP overexpression for DIV14 neurons). At DIV4, DIV7 or DIV14, hippocampal neuronal cultures were briefly stimulated with 10 µM glutamate for 30 s to induce neuronal activity in the glutamatergic system. Subsequent determination of p-ERK2 and p-CREB1 levels revealed that hippocampal cell cultures treated with 100 nM His-EGFP-sAPPα from DIV2 to DIV7 show significantly higher levels of phosphorylated ERK2 (78.4% increase, $p = 0.02$) (Fig. 3B) and CREB1 (300.1% increase, $p = 0.01$) (Fig. 3C) determined by Western blot (normalized to total levels or ERK2 or CREB1 respectively). By contrast, we did not see changes induced by His-EGFP-sAPP administration upon glutamate stimulation of DIV4 or DVI14 neuronal cell

cultures compared to treatment with the His-EGFP control peptide (Fig. 3B, C). To further elucidate the short-term effects of His-EGFP-sAPPα and His-EGFP-sAPPβ on the phosphorylation of CREB, SH-SY5Y cells were transfected with a FRET-based CREB1 biosensor (ICAP) and pre-treated with 1nM His-EGFP-sAPPα and His-EGFP-sAPPβ. Ratiometric analysis of the FRET fluorophores revealed a significant increase in CREB1 phosphorylation upon His-EGFP-sAPPα treatment 5 mins after glutamate stimulation (Fig. S3 A). CREB1 phosphorylation level was unchanged upon His-EGFP-sAPPβ treatment (Fig. S3 B).

sAPP overexpression led to advanced glutamatergic architecture at DIV14 comparable to control treated DIV21 neurons but it suppresses the formation of GABAergic inputs

sAPP effects may be limited to a subpopulation of synaptic terminals. We therefore asked to what extent glutamatergic, as opposed to GABAergic, synaptic terminals benefited from the presence of sAPPs in supernatant of neuronal culture medium. To answer this question, we transduced PHNs at DIV2 with mCherry-sAPPα-, mCherry-sAPPβ- or a mCherry-control-lentivirus. At DIV14 and DIV21, we counted VGlut1 positive puncta along the dendrite as markers for the excitatory presynapse (Fig. 4A). VGAT1 was used as a marker for inhibitory synapses (Fig. 4C). Upon mCherry-sAPPα overexpression, DIV14 PHNs showed a significant increase in VGlut1 positive puncta (20%, $p < 0.0001$) normalized to 10 µm dendrite length compared to overexpression of the mCherry control construct (Fig. 4B). When mCherry-sAPPβ was overexpressed in primary neurons until DIV14, the median number of VGlut1 positive puncta was elevated by 20% ($p < 0.0001$). The median quantity of VGlut1 positive puncta at DIV14 was 1.5 (1.2 – 1.7) in the control, 1.8 (1.5–2.1) in the mCherry-sAPPα overexpressing neurons and 1.8 (1.5 – 2.1) upon mCherry-sAPPβ overexpression in primary hippocampal neurons.

In contrast to glutamate-releasing presynapses, GABA-releasing terminals are inhibitory in function. To test whether synaptogenesis in general is elevated in the presence of exogenous sAPP, we next determined the number of GABA- versus glutamate-releasing terminals in DIV14 neurons in mCherry-sAPP-overexpressing hippocampal neurons (Fig. 4C). Upon mCherry-sAPPα overexpression, 32% less GABAergic terminals were developed compared to overexpression of the control construct ($p < 0.0001$). The number of GABA-releasing presynapses was also significantly reduced by 28% when developing neurons were exposed to

484 mCherry-sAPPβ compared to the control peptide ($p < 0.0001$) (Fig. 4D). The median of
485 absolute inhibitory presynapses were 1.0 (0.8–1.2) after mCherry-sAPPα, 1.0 (0.8–1.3) after
486 mCherry-sAPPβ and 1.4 (1.2–1.7) upon mCherry-control construct overexpression. The
487 calculated ratio of glutamatergic to GABAergic synaptic terminals was significantly increased
488 by 76% upon mCherry-sAPPα and 72% after mCherry-sAPPβ overexpression ($p < 0.001$) (Fig.
489 4E).

490 To further test the hypothesis that neuronal development *in vitro* is advanced resulting from a
491 neuronal network exposed to elevated sAPP levels, we transduced PHNs at DIV2 with a
492 mCherry-control-lentivirus and counted VGlut1 and VGAT at DIV21 and compared them with
493 DIV14 mCherry-sAPP-overexpressing neurons. We found that neurons overexpressing the
494 control construct from DIV2 to DIV21 showed 27% ($p < 0.0001$) more VGlut1, but no
495 significant change in the number of VGAT positive puncta compared to neurons overexpressing
496 the control construct from DIV2 to DIV14 (Fig. 4B,D). The median count of VGlut1 positive
497 puncta at DIV21 was 1.9 (1.6–2.2) (Fig. 4B) and 1.6 (1.3–1.9) (Fig. 4D) for VGAT positive
498 puncta upon overexpression of the control construct. A proposed model of how sAPP could
499 affect synapse development *in vitro* is depicted in Fig. 4F.

500

501 **sAPPα is decreased in AD brains and can be detected in human synaptoneurosomes**

502 Since we were able to show that sAPPα and sAPPβ have crucial functions in the development
503 of the glutamatergic system *in vitro* and activity of the major α-secretase ADAM10 and BACE1
504 activity seem to be altered in LOAD patients, we next asked whether sAPP levels are altered in
505 homogenates and synaptoneurosomes of human *post-mortem* tissue of AD patients and control
506 subjects. To do so, we enriched synaptoneurosomes from BA41/42 of end stage brain tissue by
507 a previously reported method (Tai et al., 2012). Western blot analysis with a human sAPPα-
508 specific antibody revealed that sAPPα is significantly decreased by about 53% in brain
509 homogenate of AD patients compared to control subjects (Fig. 5A, $p = 0.007$). We further
510 observed a tendency of reduced sAPPα levels in synaptoneurosomes of AD patients (Fig. 5B,
511 $p = 0.128$). We also tested if sAPPβ is detectable in human brains with a human sAPPβ specific
512 antibody. We were not able to detect full length sAPPβ in human brain homogenates or
513 synaptoneurosomes, but cleavage fragments (Fig. S4). Therefore, we did not quantify these
514 Western blots.

Discussion

Considering that levels of the soluble APP ectodomains, released by initial ADAM-10 or BACE1 cleavage, are altered in AD patients (Cuchillo-Ibañez et al., 2015; Holsinger et al., 2004; Lannfelt et al., 1995; Palmert et al., 1990; Zetterberg et al., 2008), the natural *in vivo* function of sAPPs in the CNS are of pivotal interest.

In this study, we investigated the impact of sAPPs on the glutamatergic system and the accompanying morphological changes in hippocampal neuronal cell culture side by side. We found that both sAPPβ and sAPPα stimulate the glutamatergic system in hippocampal neurons *in vitro* and regulate neuronal development and synapse formation in a stage-dependent fashion.

Our study reports several important findings. First, both sAPPβ and sAPPα affect neuronal morphology and complexity in a stage-dependent manner in PHNs. Having structurally and functionally different processes that act as communication relay in the CNS, neurons are one of the most highly polarized cell types in nature (Takano et al., 2015). As in our study, DIV4 PHNs do not show morphological differences when analyzed by determining the axon length, entire neurite length and neurite number. Therefore, sAPPs should not interfere with early stages in neuronal development *in vitro*. The observation that His-EGFP-sAPPβ-treated neurons showed different effects in Sholl analysis compared to His-EGFP-sAPPα-treated neurons, suggests that different receptors may be involved in mediating sAPPα and sAPPβ effects. Considering that, in our study, sAPPs interfered with intermediate stages (DIV7), but not with early stages (DIV4) of neuronal development in cell culture, we can assume that sAPPs affect dendritic development rather than axon specification. Since we further showed that sAPPs impact on glutamate signaling, it seems likely that functions are mediated by modulation of a subset of distinct glutamate receptors that are differentially expressed during PHN development *in vitro* (Janssens and Lesage, 2001) and *in vivo* (Luján et al., 2005). A recent study by Hasebe et al. revealed that sAPPα promotes neurite outgrowth in stage 1 neurons (24 h after plating) (Hasebe et al., 2013). This finding contradicts our results to some extent, but differences might be explained by the use of another neuronal culture system (cortical neurons instead of hippocampal neurons). In fact, in line with our findings, Chasseigneaux et al. were able to demonstrate that sAPPα increases axon length in primary neurons (Chasseigneaux et al., 2011). In contrast to our findings, they observed a decrease in dendrite outgrowth. The difference could be explained by the earlier stage (DIV5 neurons from E16 mice vs. DIV7 neurons from E18 mice) they examined or the investigation of cortical neurons instead of hippocampal neurons and the utilization of another mouse strain (Swiss instead of C57BL/6).

Our results are also in agreement with a previous study showing that sAPPα enhances axon growth (Young-Pearse et al., 2008).

Second, in this study, we show that mCherry-sAPP overexpression in PHNs results in increased glutamatergic synaptic architecture in DIV14 neurons. For precise functioning of the mammalian brain, it is crucial that neuronal contacts are formed properly and maintained in the long term, if needed. Since synaptogenesis in PHNs is dependent on neuronal activity and connectivity, amongst others, we next asked whether long-term exposure of sAPPs to PHNs impacts synaptogenesis and the formation of subsynaptic structures like the PSD. In line with our findings, that both mCherry-sAPPα and mCherry-sAPPβ overexpression led to a higher number of mature glutamatergic synapses in PHNs, Bell et al. reported that ADAM-10-overexpression in mice increased the brain sAPPα levels and the number of presynaptic bouton densities (Bell et al., 2008). Other work showed that infused sAPPα increased memory retention and synaptic density in the frontoparietal cortex of rats (Roch et al., 1994). The process of synapse formation involves axo-dendritic contact after axon extension, presynaptic and postsynaptic differentiation, synaptic maturation, pruning and maintenance (Johnson-Venkatesh and Umemori, 2010). At which stage sAPPs enhance synaptogenesis is not clear yet and remains to be answered. The observation that mCherry-sAPPα exposure leads to increased layer thickness of PSDs in PHN synapses, agrees with several studies investigating the effects of sAPPα on synaptic transmission and LTP (Taylor et al., 2008) or the ability of sAPPα to rescue synaptic deficits in different APP transgenic mice (Fol et al., 2015; Hick et al., 2015; Ring et al., 2007). Claasen et al. further showed, that sAPPα upregulates synaptic protein synthesis by a proteinkinase G-dependent mechanism in synaptoneurosomes prepared from rat hippocampi (Claasen et al., 2009). Based on these results, we can conclude that the sAPP-induced extended neuritic arborization at DIV7 ends up in an advanced synaptic architecture of glutamatergic synapses at later stages (DIV14) of neuronal development *in vitro*.

Third, we demonstrated that increased neuritic arborization is associated with higher levels of activated markers of neuronal activity. Development of the neuritic arbor and synaptogenesis are, *inter alia*, dependent on neuronal activity. To that end, we tested the hypothesis that the more sophisticated arborization at DIV7 and the advanced synaptic architecture at DIV7 upon sAPP exposure is associated with neuronal activity. We first tested whether sAPPs influence glutamate-evoked Ca^{2+} signals in mature PHNs. By showing that both His-EGFP-sAPPα and His-EGFP-sAPPβ (15 min preincubation) increase free intracellular Ca^{2+} levels upon glutamate stimulation, we concluded that sAPPs modulate glutamate sensitivity of PHNs in a short-term

response. Given that a 15 min preincubation with sAPPs was enough to alter glutamate sensitivity of PHNs, a mobilization of the synaptic AMPA-R pool mediated by sAPPs is more likely than a transcription-based response. To obtain a more detailed picture of how sAPPs modulate neuronal plasticity on a molecular level, we next asked whether glutamate-activated synaptic plasticity-dependent downstream targets of Ca^{2+} signaling are also affected by His-EGFP-sAPP exposure to neurons. Downstream components of Ca^{2+} are, amongst others, mitogen-activated protein kinase 1 (ERK2) and the transcription factor cyclic AMP-responsive element-binding protein 1 (CREB1). Hence, we tested whether His-EGFP-sAPPs modulate ERK2 and CREB1 phosphorylation in glutamate stimulated PHNs at different stages of development (DIV4, DIV7, DIV14). When PHNs were briefly stimulated with glutamate, we detected increased levels of p-ERK2 as well as p-CREB1 upon long-term His-EGFP-sAPPα exposure to neurons until DIV7. By contrast, we did not see any differences in p-ERK2 or p-CREB1 levels at DIV4 or DIV14 glutamate-stimulated PHNs when exposed to His-EGFP-sAPPs compared to control. We concluded that a peak level of phosphorylated LTP-dependent kinases at DIV7 leads to increased neuritic arborization and subsequently to an advanced development of glutamatergic synapses. Unchanged levels of activated ERK2 and CREB1 at DIV4 upon His-EGFP-sAPP treatment are associated with unaltered neuritic morphology. Long-term His-EGFP-sAPP exposure to neurons did not affect p-ERK2 or p-CREB1 levels when the neuronal network was stimulated at DIV14. The latter could be a ceiling effect or a compensatory mechanism to protect neurons from overexcitation. To determine short term effects of sAPPs, we analyzed time resolution of sAPP treatment immediately after treatment in SH-SY5Y cells. We found an immediate response on CREB1 phosphorylation upon His-EGFP-sAPPα treatment. These results are in line with a study by Ryan et al. showing that sAPPα induces time-dependent changes in gene expression in the rat hippocampus, amongst other downstream targets of CREB1 (Ryan et al., 2013). Our results further confirm recent findings revealing that sAPPα and sAPPβ promote Egr1/ERK1 signaling leading to enhanced axon outgrowth *in vitro* (Chasseigneaux et al., 2011). Moreover, a study by Rohe et al. showed that increased levels of sAPP correlated with pro-found stimulation of neuronal ERK signaling (Rohe et al., 2008). Since we have shown that sAPPs modulate glutamate induced CREB1 activation, a direct modulation of one or more distinct glutamate receptor seem to be likely. However, which of glutamate receptor (or subunit composition) is modulated by sAPPs remains to be determined. At least, one study by Taylor et al. showed that sAPPα regulates hippocampal NMDA-receptor function (Taylor et al., 2008).

Fourth, mCherry-sAPP overexpression led to advanced glutamatergic architecture at DIV14 comparable to control-treated DIV21 neurons, but it suppresses the formation of GABAergic inputs. Since we showed, that both sAPPs positively regulate neuritic arborization and synapse formation, we determined whether the sAPP effects are a mere mechanism of advanced development. Therefore, we compared the number of inhibitory and excitatory synaptic inputs of mCherry-sAPP-treated PHNs at DIV14 with control treated DIV21 neurons. We found that the number of excitatory synaptic inputs of DIV14 PHNs exposed to mCherry-sAPPs was comparable with control-treated DIV21 neurons. In contrast, the number of inhibitory inputs in hippocampal cell culture was not decreased in the control when comparing DIV21 with DIV14. We show here for the first time that the number of inhibitory synaptic inputs, determined by VGAT immunostaining, is decreased when comparing control treated DIV14 inhibitory inputs with DIV14 mCherry-sAPP-treated inhibitory inputs. Therefore, we concluded that sAPP effects are not merely resulting from advanced neuronal development, but rather modulate the ratio of excitatory to inhibitory inputs. Since proper formation and function of neuronal circuits depends on the balance of inhibitory and excitatory mechanisms, this is an important finding. To rule out that the decrease in GABAergic input is a consequence of cell death, we tested if sAPPs impact on cell viability by the MTT assay. We found no impact of sAPP preparations on cell viability, even at high concentrations (data not shown). Interestingly, genes related to GABAergic signaling like glutamate decarboxylase 1 and 2 (GAD) or somatostatin (Sst) possess CREB1 binding sites in their promotor regions (Zhang et al., 2005). Furthermore, in patients suffering from autism spectrum disorders (ASDs), an imbalance in GABAergic/glutamatergic inputs was observed (El-Ansary and Al-Ayadhi, 2014) and increased levels of sAPPα were described in the CSF of ASD patients (Ray et al., 2011). Whether these findings correlate statistically significantly or are causative for pathophysiology remains to be determined. Our finding that neuronal complexity, synapse maturation and E/I balance are increased in PHNs upon sAPP exposure are in line with several studies focusing on BDNF effects on developing neurons (Ji et al., 2010; Singh et al., 2006). This raises the question of whether sAPP can directly/indirectly modulate or bind to TrkB receptors to induce BDNF-like effects. Further studies are needed to shed light on this hypothesis.

Fifth, in almost all our assays assessing the effects of sAPPs on glutamatergic development, we observed that sAPPβ effects are similar to neurotrophic sAPPα effects. These findings disprove our hypothesis that sAPPβ differs from the neurotrophic effects on the developing glutamatergic system of sAPPα. Differences between sAPPα and sAPPβ were seen in the Sholl analysis that revealed a distinct pattern of ramification for sAPPα and sAPPβ, with increased

branching close to the soma for sAPPα. In contrast to sAPPα-treated neurons, sAPPβ exposure did not lead to axon elongation. Furthermore, sAPPβ-treated neurons did not show increased PSD layer thickness or ERK phosphorylation, whereas sAPPα-treated neurons did. Interestingly, a study by Furukawa et al. could show that sAPPα was approximately 100-fold more potent than sAPPβ in protecting hippocampal neurons against excitotoxicity, Aβ toxicity, and glucose deprivation (Furukawa et al., 1996b). The main domains described to mediate the neurotrophic effects of sAPPα are shared by sAPPβ, except for the last 16 c-terminal amino acids. This would favor a model where the common parts of sAPPα and sAPPβ mediate the observed functions. Other functions might be affected differentially as a study by Peters-Libeu et al. indicated that, despite the large sequence homology between both sAPPs, the folding is different, leading to different effects on APP cleavage by BACE1 (Peters-Libeu et al., 2015). If sAPPα and sAPPβ bind (e.g. depending on their folding) to the same or distinct receptors to mediate their functions remains elusive.

Finally, we observed that sAPPα is significantly decreased in human AD brains and detectable in human synaptoneurosomes compared to control subjects. Until now, almost all studies investigating the protein levels of sAPPs in human CNS used CSF as sample material (Colciaghi et al., 2004; Hock et al., 1998; Lannfelt et al., 1995; Palmert et al., 1990). We show here for the first time, that sAPPα is significantly decreased in AD brains and abundant in human synaptoneurosomes in AD patients and control subjects. Moreover, we observed a reduction in sAPP levels in human synaptoneurosomes from AD patients compared to control subjects by trend. Both confirmed studies which found decreased sAPPα levels in CSF of AD patients (Colciaghi et al., 2004; Hock et al., 1998; Lannfelt et al., 1995; Palmert et al., 1990). The sAPPβ antibody used in our study was not suitable to detect full length sAPPβ. For this reason, it remains to be determined if sAPPβ is also altered in AD brains. Since we demonstrated several crucial effects of sAPPα and sAPPβ in neuronal development, reduced sAPPα levels in brains of AD patients could have deleterious effects beyond Aβ pathophysiology, e.g. due to decrease of specific neurotrophic or neuroprotective mechanisms. Furthermore, altered sAPP levels in the developing brain might contribute to selective vulnerability of hippocampal neurons and therefore accelerating disease progression, e.g. by interfering with neuronal cell-type specification mechanisms (Götz et al., 2009). Disturbances in adult neurogenesis might be another explanation of how altered sAPP levels can account for AD pathophysiology (Lazarov and Demars, 2012; Lazarov and Marr, 2010; Wang et al., 2014).

Taken together, our study confirmed previous findings describing neurotrophic effects for sAPPα and revealed that human recombinant sAPPβ effects resemble sAPPα effects, but to a lesser extent. We further showed that neurotrophic effects of both sAPPs in early PHN development are associated with advanced glutamatergic development and revealed that sAPPs lead to altered E/I balance in mature hippocampal neurons (Fig. 4F). Synaptic impairments observed in AD patients might be a consequence not only of Aβ accumulation, but also to the loss of synapse-promoting abilities of sAPPα and sAPPβ. Our data indicate critical stage-dependent roles of both sAPPs in the developing glutamatergic system *in vitro*, and thus lead to a better understanding of deleterious consequences of altered APP shedding in AD patients and BACE-1 inhibitor recipients.

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Ethics approval and consent to participate: Use of human tissue for post-mortem studies has been reviewed and approved by the Edinburgh Brain Bank ethics committee and the ACCORD medical research ethics committee, AMREC (ACCORD is the Academic and Clinical Central

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Figure Legends

Figure 1: **Morphological analysis of primary hippocampal neurons upon His-EGFP-sAPP treatment at DIV4 and DIV7.** DIV2 PHNs were treated with 100nM His-EGFP-sAPPα or His-EGFP-sAPPβ and fixed and stained at DIV4 (A-C) or DIV7 (D-H). A) Boxplots comparing primary axon lengths of PHNs treated with control peptide and His-EGFP-sAPP treated neurons. Statistical analysis revealed that there is no statistically significant difference between

His-EGFP-control and His-EGFP-sAPP treated groups. $p < 0.05$, One-Way ANOVA, Kruskal-Wallis Test. B) Boxplots comparing entire neurite lengths of PHNs treated with His-EGFP-control peptide and His-EGFP-sAPP treated neurons. Statistical analysis revealed that there is no statistically significant difference between control and His-EGFP-sAPP treated groups. $p < 0.05$, One-Way ANOVA, Kruskal-Wallis Test. C) Boxplots comparing neurite numbers of PHNs treated with His-EGFP-control peptide and His-EGFP-sAPP treated neurons. Statistical analysis revealed that there is no statistically significant difference between His-EGFP-control and His-EGFP-sAPP treated groups. $p < 0.05$, One-Way ANOVA, Kruskal-Wallis Test. D) Boxplots comparing primary axon lengths of PHNs treated with His-EGFP-control peptide and His-EGFP-sAPP treated neurons. Statistical analysis revealed that there is a statistically significant difference between His-EGFP-control and His-EGFP-sAPPα treated group. $p < 0.05$, One-Way ANOVA, Kruskal-Wallis test. E) Boxplots comparing entire neurite lengths of PHNs treated with His-EGFP-control peptide and His-EGFP-sAPP treated neurons. Statistical analysis revealed that there is a statistically significant difference between His-EGFP-control and His-EGFP-sAPPα as well as His-EGFP-sAPPβ treated groups. $p < 0.05$, One-Way ANOVA, Kruskal-Wallis Test. F) Boxplots comparing neurite numbers of PHNs treated with His-EGFP-control peptide and His-EGFP-sAPP treated neurons. Statistical analysis revealed that there is a statistically significant difference between His-EGFP-control and His-EGFP-sAPPα as well as His-EGFP-sAPPβ treated groups. $p < 0.05$, One-Way ANOVA, Kruskal-Wallis Test. G) Representative diagram view of sholl analysis of dendritic complexity from PHNs. H) Sholl analysis of dendritic complexity of PHNs treated with His-EGFP-control peptide or His-EGFP-sAPPα (upper plot) / His-EGFP-sAPPβ (lower plot). Each group comprises N=60 neurons from 3 independent cultures (~180 neurons in total for Fig. 1 A-F and ~60 neurons in total for Fig. 1 H). Asterisks indicate a statistically significant difference between His-EGFP-control and His-EGFP-sAPP treated groups. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, position wise Wilcoxon-test (26 tests, Bonferroni correction for multiple testing). Overall differences of curves are tested with the CuCompare software.

Figure 2: **Advanced glutamatergic architecture upon mCherry-sAPP overexpression.** PHNs were lentiviral transduced at 2DIV with either mCherry-control, mCherry-sAPPα or mCherry-sAPPβ virus. A) Exemplary hippocampal neuron picture of mCherry-control, mCherry-sAPPα and mCherry-sAPPβ transduced neurons. Neurons were fixed at DIV14 and stained with Homer1 (red) and Bassoon (green) antibodies. Colocalizations indicating a mature glutamatergic synapse were counted and normalized to 10µm dendrite length. Scale bar: 10µm B) Statistical analysis revealed a significant higher number of Homer1/Bassoon colocalizations

upon mCherry-sAPPα as well as mCherry-sAPPβ overexpression. $p < 0.0001$, Kruskal-Wallis test. 180 neurons from 3 independent cultures were analyzed for each condition. C) Analysis of Homer1/Bassoon colocalizations after mCherry-control, mCherry-sAPPα or mCherry-sAPPβ overexpression (plotted according to cumulative frequency) revealed that mCherry-sAPPα as well as mCherry-sAPPβ lead to a homogenous rightward shift in the frequency curve. D) Exemplary images of synaptic ultrastructure of mCherry-control, mCherry-sAPPα and mCherry-sAPPβ overexpressing hippocampal DIV14 neurons obtained by transmission electron microscopy. Asterisks indicate the postsynaptic density. Scale bar: 100 nm. E) Statistical analysis revealed a significant increase of postsynaptic density layer thickness of mCherry-sAPPα exposed neurons. $p < 0.0001$, Kruskal-Wallis test. 60 PSDs from three independent cultures were analyzed for each condition F) Analysis of layer thicknesses of PSDs after mCherry-control, mCherry-sAPPα or mCherry-sAPPβ overexpression (plotted according to cumulative frequency) revealed that mCherry-sAPPα lead to a rightward shift in the frequency curve.

Figure 3: **Glutamate sensitivity of DIV7 PHNs is increased upon His-EGFP-sAPP treatment.** A) 15 minutes pre-Incubation of PHNs with both His-EGFP-sAPPα and His-EGFP-sAPPβ (1 nM) increase the Ca^{2+}-influx in DIV14 PHNs upon glutamate (10 µM) stimulation. n=3, N=18 wells; $p < 0.05$, Two-Way ANOVA, Dunnett's post hoc test. B-C) DIV2 PHNs were treated with 100nM His-EGFP-sAPPα or His-EGFP-sAPPβ and stimulated with 10 µM glutamate at DIV4, DIV7 or DIV14 respectively. To determine phospho ERK2 (lower bands) and phospho CREB1 levels Western blots were quantified by densitometric analysis. p-ERK2 levels were normalized to t-ERK2 levels and p-CREB1 levels were normalized to total levels of CREB1 B) Relative protein levels of p-ERK2 were significantly increased upon His-EGFP-sAPPα treatment at DIV7. $p = 0.02$ Kruskal-Wallis Test. n=3 independent cultures. p-ERK2/t-ERK2 ratio was not significantly altered upon His-EGFP-sAPP treatment and glutamate stimulation at DIV4 and DIV14. C) Relative protein levels of p-CREB1 were significantly increased upon His-EGFP-sAPPα treatment and increased by trend after His-EGFP-sAPPβ treatment at DIV7. His-EGFP-sAPPα vs His-EGFP-control: $p = 0.001$, His-EGFP-sAPPβ vs. His-EGFP-control: $p = 0.056$. Kruskal-Wallis test. n=3 independent cultures, at least three technical replicates per culture. P-CREB1/t-CREB1 ratio was not significantly altered upon His-EGFP-sAPP treatment and glutamate stimulation at DIV4 and DIV14.

Figure 4: **mCherry-sAPP overexpression lead to advanced glutamatergic architecture at DIV14 comparable to control treated DIV21 neurons but it suppresses the formation of**

GABAergic inputs. PHNs were lentiviral transduced at DIV2 with either mCherry-control, mCherry-sAPPα or mCherry-sAPPβ virus. mCherry-sAPP treated neurons were fixed and stained at DIV14. mCherry-Control treated neurons were fixed and stained and DIV14 and DIV21. A) Exemplary PHN dendrites after overexpression of control construct and fixed at DIV14 and DIV21 and overexpression of mCherry-sAPPα or mCherry-sAPPβ fixed at DIV14. Presynapses were stained by VGlut1 antibodies. B) Statistical analysis revealed a significant higher number VGLUT1 positive puncta (presynaptic marker) upon mCherry-sAPPα as well as mCherry-sAPPβ overexpression. PHNs overexpressing the control construct $p < 0.0001$, Kruskal-Wallis test. 60 neurons from 3 independent cultures were analyzed for each condition. C) Exemplary PHN dendrites after overexpression of control construct and fixed at DIV14 and DIV21 and overexpression of mCherry-sAPPα or mCherry-sAPPβ fixed at DIV14. Presynapses were stained by VGAT antibodies. B) Statistical analysis revealed a significant higher number VGAT positive puncta (presynaptic marker) upon mCherry-sAPPα as well as mCherry-sAPPβ overexpression. PHNs overexpressing the control construct $p < 0.0001$, Kruskal-Wallis test. 60 neurons from 3 independent cultures were analyzed for each condition. E) The E/I ratio was calculated by dividing the average number of VGlut1 pos. puncta by the average number of VGAT pos. puncta along 10 µm dendrite length. The foldchange over control was calculated and plotted. E/I ratio at DIV14 of mCherry-sAPPα as well as mCherry-sAPPβ treated neurons is significantly increased compared to mCherry-control treated neurons. When comparing DIV14 control treated neurons with DIV21 mCherry-control treated neurons, no significant change was observed. $p < 0.0001$, One-Way ANOVA, Kruskal-Wallis test, Dunn's post-hoc test. F) Schematic diagram for proposed functions of sAPPs in modulating glutamatergic development in PHNs.

Figure 5: **sAPPα levels are significantly reduced in brain homogenates of AD patients.** A) Qualitative Western blot analysis of 14 human brains (seven control and seven AD brains) revealed that sAPPα is detectable by a specific sAPPα antibodies in human brain homogenates and that sAPPα levels are significantly lower in AD brain homogenates compared to control subjects. $p = 0.007$, Mann-Whitney *U*-test. B) Enrichment of synaptic fraction showed that sAPPα are detectable in the corresponding synaptic pellets and a trend of reduced sAPPα levels in AD synaptoneurosomes compared to control subjects was observed. $p = 0.128$, Mann-Whitney *U*-test. Western blots were quantified by densitometric analysis.

Figure S1: **Qualitative analysis of recombinant His-EGFP-sAPPs produced in Flp-In-HEK293 cells and by using a lentiviral system reveals no cross contamination between**

sAPPα and sAPPβ production A) Simply blue safe stained SDS-PAGE gel showing no side bands in His-EGFP-sAPPα and His-EGFP-sAPPβ elutions (His purification from HEK293 cell supernatant). B) Corresponding Western Blot to stained SDS-PAGE gel. The upper blot shows bands detected with an anti β-Amyloid antibody (6e10). In the His-EGFP-sAPPα elution lane a band at ~120 kDa can be detected, whereas no band is visible in the His-EGFP-sAPPβ elution lane. The lower blot shows bands detected with an antibody directed against the n-terminus of APP (22c11). There are bands at ~120 kDa visible in the His-EGFP-sAPPα and His-EGFP-sAPPβ elution lane. C) Western Blots showing the supernatant of DIV14 PHNs upon sAPP lentiviral transduction at DIV2. The left blot shows a band detected with an anti β-Amyloid antibody (6e10) in the supernatant of mCherry-sAPPα transduced neurons. The right blot shows bands detected with an antibody directed against the n-terminus of APP (22c11) in the supernatant of mCherry-sAPPα and mCherry-sAPPβ transduced neurons. No band is visible in the supernatant of mCherry-pUltra hot transduced neurons. D) Schematic illustration of specificity of the used antibodies for the qualification of recombinant sAPP production.

Figure S2: **Exemplary images of DIV4 and DIV7 PHNs used for morphometric analysis upon control peptide and His-EGFP-sAPP treatment.** A-C) PHNs were treated with control peptide or His-EGFP-sAPPs at DIV2 and stained at DIV4 with an anti-beta-III-tubulin antibody to assess the morphology of His-EGFP-control (A), His-EGFP-sAPPα (B) and His-EGFP-sAPPβ (C) treatment. D-F) PHNs were treated with His-EGFP-control peptide or His-EGFP-sAPPs at DIV2 and stained at DIV7 with anti-tau (red) and anti-MAP2 antibodies (green) to assess the morphology of His-EGFP-control (D), His-EGFP-sAPPα (E) and His-EGFP-sAPPβ (F) treatment. Scale bars: 100µm.

Figure S3: **His-EGFP-sAPPα increases CREB-1 phosphorylation in SH-SY5Y cells in a time-dependent manner.** A) Ratiometric analysis of Venus and mTurquoise fluorescence intensities shows a significant increase of CREB-1 phosphorylation in SH-SY5Y cells upon His-EGFP-sAPPα pre-treatment (1nM). CREB phosphorylation was determined by the CREB-phosphorylation biosensor ICAP. Cells were stimulated with 10µM glutamate and Venus/mTurquoise intensities ratios were recorded after 5, 12, 19, 24, 30, 35, 40, 45, 50, 55 and 60 minutes. B) Ratiometric analysis of Venus and mTurquoise fluorescence intensities shows no alterations of CREB-1 phosphorylation in SH-SY5Y cells upon His-EGFP-sAPPβ pre-treatment (1nM). CREB phosphorylation was determined by the CREB-phosphorylation biosensor ICAP. Cells were stimulated with 10µM glutamate and Venus/mTurquoise intensities ratios were recorded after 5, 12, 19, 24, 30, 35, 40, 45, 50, 55 and 60 minutes. His-EGFP-control

group (N=21), His-EGFP-sAPPα (N=11), His-EGFP-sAPPβ (N=21). Asterisks indicate a statistically significant difference between His-EGFP-control and His-EGFP-sAPP α treated groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, position wise Wilcoxon-test (12 tests, Bonferroni correction for multiple testing). Overall differences of curves are tested with the CuCompare software.

Figure S4: **sAPPβ is not detectable in human brain homogenates.** Western blot showing sAPPβ cleavage fragments. Full length sAPPβ at 90-100 kDA molecular weight is not detectable.

Table S1: **Patient demographic data**

Edinburgh brain bank number	BBN NUMBER	Gender	diagnosis	Age[y]
SD001/16	28406	M	control	79
SD017/16	28793	F	control	79
SD018/16	28794	F	control	79
SD024/15	26495	M	control	78
SD051/15	19597	M	control	79
SD032/13	16425	M	control	61
SD063/13	19686	F	control	77
SD049/14	24322	M	AD	80
SD055/14	24526	M	AD	79
SD056/14	24527	M	AD	81
SD007/15	25056	M	AD	72
SD014/15	25739	F	AD	85
SD040/15	26718	M	AD	78
SD005/16	28410	F	AD	62

M = male, F = female

Supplementary Methods:

Measurement of the time-dependent phosphorylation of CREB1

To measure the time-dependent phosphorylation of CREB1 SH-SY5Y cells were transfected with a FRET based indicators of phosphorylation for CREB1 (pNLS- mTurquoise2Δ-11AA-ICAP- td_cp173Venus(d)_188_NES-pUltraHot) (Friedrich et al., 2010; Klarenbeek et al., 2015). On the day before the transfection SH-SY5Y cells were seeded onto a 96 Well Black Clear Flat Bottom TC-Treated Microplate (Corning, New York, USA) at a density of $4x10^4$

cells per well. The cells were incubated overnight at 37 ° C and 5% CO2 in 10 ml DMEM plus 10 % fetal calf serum and 1 % Penicillin with Streptomycin and are transfected the next morning according to the manufacturer's instructions of the LTX & Plus Reagent Kit. After another 24 hours of incubation under the same conditions the cells were used for measurements in the microplate reader. To carry out the experiments the medium must be replaced by a clear wash buffer (for buffer composition see FLIPR-analysis part in Material & Methods section) which does not influence the fluorescence intensity to be measured. The 96 well plate was then placed in the to 37 ° C preheated Clariostar microplate reader (BMG Labtech, Ortenberg, Germany). In the reader, the cells were excited with a wavelength of 430 nm and the emission is measured at 480 nm and 550 nm. The first emission wavelength corresponds to the range in which mTurquoise2Δ emits, the second wavelength is slightly above the emission of the Venus fluorophore (530 nm) to avoid a blend with the emitting light of turquoise. Other important measurement settings are: focal height 3.8 mm, bottom reading and well scan with a 5x5 matrix. First the fluorescence intensities of the untreated cells were measured. Subsequently, the cells were treated with glutamate (final concentration 10 μM) and the fluorescence intensity was measured again at 5, 12, 19, 24, 30, 35, 40, 45, 50, 55 and 60 minutes after stimulation for the measurements of CREB-activity. For the measurements, the cells were pretreated with His-EGFP-sAPPα/β / His-EGFP-control peptide (1nM) for 24 h. The analysis of the measured fluorescence intensities was performed with the Clariostar Data Analysis software (BMG Labtech, Ortenberg, Germany). For the evaluation, the fluorescence intensity of Venus (detection at 550 nm) was set in relation to the fluorescence intensity of Turquoise (detection at 480 nm), the ratio was then standardised to the fluorescence ratio of the untreated and unstimulated cells.